METHODS OF INDUCING OR ENHANCING CONNECTIVE TISSUE REPAIR

This application is a Continuation- in Part Application of United States Serial No. 09/376, 276, filed August 2, 1999 which claims priority from DE Application No. 198.37.438.0, filed August 18, 1998, which are both incorporated herein by reference.

FIELD OF THE INVENTION

0001 The invention relates to methods of enhancing repair of a cartilage and/or inducing formation of a cartilage by administering a cell which expresses at least one factor of the T-box family, which includes *inter-alia* the brachyury. In another embodiment, the invention relates to an engineered cell, which is transfected with a vector comprising at least one nucleic acid sequence encoding a factor of the T-box family, thereby expressing at least one factor of the T-box family. In another embodiment, the invention relates to compositions comprising a vector which comprises at least one nucleic acid sequence encoding a factor of the T-box family and in another embodiment the composition comprising cell which expresses at least one factor of the T-box family, which includes *inter-alia* the brachyury.

BACKGROUND OF THE INVENTION

0002 The meniscus, fibrocartilaginous tissue found within the knee joint, is responsible for shock absorption, load transmission, and stability within the knee joint. According to the National Center for Health Statistics, over 600,000 surgeries each year are the result of complications with the meniscus. The meniscus has the intrinsic ability to heal itself; unfortunately, this property is limited only to the vascular portions of the tissue. For damage outside of these areas and overall degeneration of the tissue, methods need to be developed that will assist the meniscus in healing itself Sweigart MA Tissue Eng 7(2), 111-29 (April 2001).

0003 Degeneration of articular cartilage in osteoarthritis is a serious medical problem caused by arthritis, both rheumatoid and osteoarthritis. Drugs are given to control the pain and to keep the swelling down, but the cartilage continues to be destroyed. Eventually, the joint must be replaced. It is still unknown why cartilage does not heal and no solutions to this

problem are known Mankin, N.E. J. Med. 331(14), 940-941 (October 1994).Soon after superficial injury, chondrocytes adjacent to the injured surfaces show a brief burst of mitotic activity associated with an increase in glycosaminoglycan and collagen synthesis. Despite these attempts at repair, there is no appreciable increase in the bulk of cartilage matrix and the self repair process is usually ineffective in healing the defects.

0004 Osteochondral, or full-thickness, cartilage defects expand into the subchondral bone. Such defects arise after the detachment of osteochondritic dissecting flaps, fractured osteochondral fragments, or from chronic wear of degenerative articular cartilage. Osteochondral defects depend on the extrinsic mechanism for repair. Extrinsic healing relies on mesenchymal elements from subchondral bone to participate in the formation of new connective tissue. This fibrous tissue may or may not undergo metaplastic changes to form fibrocartilage. Even if fibrocartilage is formed, it does not display the same biochemical composition or mechanical properties of normal articular cartilage or subchondral bone and degenerates with use, Furukawa, et al., J. Bone Joint Surg. 62A, 79 (1980); Coletti, et al., J. Bone Joint Surg. 54A, 147 (1972); Buckwalter, et al., "Articular cartilage: composition, structure, response to injury and methods of facilitating repair", in Articular Cartilage and Knee Joint Function: Basic Science and Arthroscopy, Ewing J E, Ed., (New York, Raven Press, 1990), 19.

0005 Injection of dissociated chondrocytes directly into the site of the defect has also been described as a means for forming new cartilage, as reported by Brittberg, et al., N. E. J. Med. 331, 889-895 (October 1994). Cartilage was harvested from minor load-bearing regions on the upper medial femoral condyle of the damaged knee, cultured, and implanted two to three weeks after harvesting.

0006 Moreover, if the defect includes a part of the underlying bone, this is not corrected by the use of chondrocytes. The bone is required to support the new cartilage.

0007 Cartilage grafts are also needed in plastic surgery like in rhinoplasty, and the reconstruiction of ears.

0008 The possibility of using stem cells was also examined. Stem cells are cells which are not terminally differentiated, which can divide without limit, and divide to yield cells that are either stem cells or which irreversibly differentiate to yield a new type of cell. Unfortunately, there is no known specific inducer of the mesenchymal stem cells that yields only cartilage. In

vitro studies in which differentiation is achieved using different bioactive factors or molecules, yields differentiation of the cells to cartilage which eventually calcified and turned into bone.

0009 Thus, there is a need to have a method and composition for the formation or repair of a cartilage or a bone. In another embodiment, it will be highly advantageous to have a cell which can divide and form a cartilage or a bone tissue.

SUMMARY OF THE INVENTION

0010 In one embodiment the invention provides a method of enhancing repair of a cartilage comprising the step of administering to a subject an effective amount of a cell which expresses at least one factor of the T-box family, thereby enhancing repair of the cartilage.

0011 In another embodiment the invention provides a method of inducing formation of a cartilage comprising the step of administering to a subject an effective amount of a cell which expresses at least one factor of the T-box family, thereby inducing formation of the cartilage.

0012 In another embodiment the invention provides a method of enhancing repair of a cartilage in the body comprising the step of administrating a recombinant vector which comprises a nucleic acid encoding a factor of the T-box family to the cartilage of a subject, thereby enhancing repair of the cartilage.

0013 In another embodiment the invention provides a method of inducing formation of a cartilage in the body comprising the step of administrating a recombinant vector which comprises a nucleic acid encoding a factor of the T-box family to the cartilage of a subject, thereby inducing formation of the cartilage.

0014 In another embodiment the invention provides a method of inducing chondrocyte differentiation comprising the step of administering of a recombinant vector which comprises a nucleic acid encoding a factor of the T-box family, thereby inducing chondrocyte formation.

0015 In another embodiment the invention provides a method of repairing or forming a cartilage in a subject in need comprising the steps of: obtaining a cell from of the subject;

0016 transfecting said cell with a recombinant vector comprising a nucleic acid sequence encoding a factor of the T-box family, so as to obtain an engineered cell which expresses a factor of the T-box family; and administering said engineered cell to the subject.

0017 In another embodiment the invention provides a method for the production of transplantable cartilage matrix, the method comprising the steps of: obtaining a cell;

transfecting said cell with a recombinant vector comprising a nucleic acid sequence encoding a factor of the T-box family, so as to obtain an engineered cell which expresses a factor of the T-box family; and culturing said cell with the cell-associated matrix for a time effective for allowing formation of a transplantable cartilage matrix.

0018 In another embodiment the invention provides an engineered cell which expresses a factor of the T-box family.

0019 In another embodiment the invention provides an implant device comprising at least one engineered cell which expresses a factor of the T-box family and a pharmaceutically acceptable carrier.

0020 In another embodiment the invention provides a composition comprising an engineered cell which expresses a factor of the T-box family and a pharmaceutically acceptable carrier.

0021 In another embodiment the invention provides a composition comprising at least one recombinant vector which comprises a nucleic acid sequence encoding at least one factor of the T-box family and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

0022 **Fig. 1.** FGFR3 mediates chondrocytic differentiation in mesenchymal stem cell line C3H10T1/2. Fig 1a shows the RT-PCR analyses of BMP2-dependent expression of FGF- and PTH/PTHrP-receptors in mesenchymal stem cell line C3H10T1/2 in the presence or absence of recombinantlly expressed BMP2. Fig 1b shows the effect of cyclohexamide pretreatment of C3H10T1/2 cells. Cycloheximide pre-treatment of C3H10T1/2 cells does not prevent BMP-induction of the FGFR3 gene. Cells were mock-treated (control) or were treated with BMP2 (50 ng/ml). Fig 1c demonstrates western immunoblotting for the detection of BMP2-dependent FGFR3 and FGFR2 expression in cellular extracts of C3H10T1/2 lines. Fig 1d *left panel:* western immunoblotting to demonstrate the forced expression of FGFR3 in mesenchymal progenitors C3H10T1/2; *right panel:* the recombinant expression of FGFR3 in C3H10T1/2 leads to enhanced levels of activated MAP-kinases pERK-1 and pERK-2 during cultivation. Cell lysates were prepared 0 (=confluence) and 4 days post-confluence. Fig 1e demonstrates that the forced expression of FGFR3 in parental C3H10T1/2 cells is sufficient for the induction of the chondrogenic lineage.

0023 **Fig. 2.** The T-box transcription factor *Brachyury* mediates chondrogenic differentiation in MSCs *in vitro* and ectopically *in vivo*. Fig 2a upper panel: schematic representation of *Brachyury* according to Kispert et al., 1995 (1995). Fig 2a lower panel: western immunoblotting of recombinant HA-tagged *Brachyury* (aa 1-436) in cellular extracts of C3H10T1/2 (C3H10T1/2-*Brachyury*) with HA-antibody SC-805 (Santa Cruz) *Brachyury* has been constitutively expressed under the control of the murine PGK-promoter. Expression of *Brachyury* is indicated (triangle). Molecular weight marker (M) shown is ovalbumin (43 kDa). Fig 2b shows the histological characterization of C3H10T1/2-*Brachyury* cells in culture. *Upper panel:* at day 4 post-confluency cells develop alkaline-phosphatase positive osteoblast-like cells. *Lower right panel*: Alcian Blue histology of C3H10T1/2 cells stably expressing *Brachyury* indicative for secreted proteoglycans and efficient differentiation into the chondrogenic lineage. *Lower left panel:* Collagen-immunohistochemistry of C3H10T1/2-*Brachyury* cells in culture 7 days post-confluency. Fig 2c shows the RT-PCR analysis of the expression of chondrogenic and osteogenic marker genes in C3H10T1/2 cells recombinantlly-expressing *Brachyury*. Fig 2d shows the forced expression of the T-box factor *Brachyury* in C3H10T1/2 cells which leads to differentiation into chondrocytes and cartilage development at murine ectopic sites after intramuscular transplantation.

0024 **Fig. 3.** Dominant-negative *Brachyury* (dn*Brachyury*; T-box domain) blocks BMP2-mediated chondrogenic development in C3H10T1/2 MSCs *in vitro* and ectopically *in vivo*. Fig 3a shows that *Brachyury's* T-box domain interferes with the transcriptional activity of full-length *Brachyury*. Fig 3b shows expression of dn*Brachyury* (T-box domain) in C3H10T1/2-BMP2 during cultivation (day 0; cellular confluence) The T-box domain (aa 1-229) has been subcloned and HA-tagged in expression vector pMT7T3 and constitutively expressed in C3H10T1/2-BMP2 cells. The recombinantlly expressed T-box domain (dn*Brachyury*) is indicated (triangle). Fig 3c demonstrates RT-PCR experiments with osteo-/chondrogenic marker genes show that T-box domain (dn*Brachyury*) expression in C3H10T1/2-BMP2 cells interferes with the BMP2 dependent of FGFR2 but not FGFR3 expression. Fig 3d shows that dn*Brachyury* (T-box) interferes with BMP2-mediated FGFR2 expression as analyzed by western immunoblotting with antiFGFR3 and antiFGFR2 antibodies as described Fig. 1. Fig 3e shows the forced expression of the dominant-negative

acting T-box domain in C3H10T1/2-BMP2 cells interferes with BMP-2 mediated osteo-/chondrogenic development.

0025 **Fig. 4.** Dominant-negative FGFR3 (dnFGFR3) interferes with osteo-/chondrogenic development, with FGFR2- and with *Brachyury*-expression in C3H10T1/2-BMP2. Fig 4a shows that forced expression of dnFGFR3 in C3H10T1/2-BMP2 cells interferes with BMP-2 mediated development of alkaline phosphatase positive and Alcian Blue positive chondrocyte-like cells, respectively. Fig 4b shows that dnFGFR3 interferes with BMP2-dependent FGFR2 and *Brachyury* but not with FGFR3 expression in C3H10T1/2-BMP2 cells.

0026 **Fig. 5.** FGFR3 and *Brachyury* are involved in an autoregulatory loop. Fig 5a shows RT-PCR analyses of FGFR3 and *Brachyury* mRNA levels in mesenchymal progenitors C3H10T1/2 expressing recombinant FGFR3 (C3H10T1/2-FGFR3) or *Brachyury* (C3H10T1/2-*Brachyury*). Fig 5b shows that Smad1-signaling is not sufficient for *Brachyury* and FGFR3 but for osteocalcin expression. RT-PCR analyses of FGFR3 and *Brachyury* mRNA levels in mesenchymal progenitors C3H10T1/2 expressing the biologically active Smad1-MH2 domain (C3H10T1/2-Smad1-MH2).

0027 **Fig. 6.** Brachyury is expressed at skeletal sites during late murine embryonic development (18.5 dpc). Comparative expression analysis of murine *Brachyury* (Bra), Collagen 1a1 (Col 1a1) and Collagen 2a1 (Col 2a1) in embryonic development 18.5 dpc. a, Intervertebral discs development. Consecutive sagittal (a-g) and transversal (h-j) sections of 18.5 dpc mouse embryos were hybridized with riboprobes as indicated. Expression of *Brachyury* is enhanced in the nucleus pulposus (a, d), Col 1a1 in the outer annulus (arrowheads in b, e), and Col 2a1 in the cartilage primordium of the vertebrae (c,f). No signals are obtained using RNase pre-incubated sections (g). With transversal sections at the level of the upper lumbar vertebra expression of *Brachyury* is in addition detectable in distinct cells of the neural arch (h) whereas Col 1a1 is expressed in the outer annulus (i) and Col 2a1 in the cartilage primordium (j). b, Limb bud development. Consecutive transversal sections of a 18.5 dpc mouse hind limb at the level of the metatarsals hybridized with riboprobes as indicated. Expression of *Brachyury* is evident in distinct chondrogenic cells of the forming metatarsal bones (a), better visible with higher magnification (b,c). In contrast Col 1a1 is expressed in

the outer periosteal layer (d-f) and Col 2a1 expression is enhanced in differentiating chondrocytes (g-i). As it was evident for the intervertebral disc formation expression of *Brachyury* is only evident in chondrocyte-like cells that do not express Col 2a1. ch, chondrocytes; cp, cartilage primordium; mta, metatarsal; np, nucleus pulposus; oa, outer annulus; pl, periosteal layer; sk, skin bar, 100□m

0028 **Fig. 7.** Model of BMP2-dependent osteo-/chondrogenic development in mesenchymal stem cells.

DETAILED DESCRIPTION OF THE EMBODIMENTS

0029 The invention relates to methods of enhancing repair of a cartilage and/or inducing formation of a cartilage by administering a cell which expresses a factor of the T-box family, which includes *inter-alia* the brachyury. In another embodiment, the invention relates to an engineered cell, which is transfected with a vector comprising a nucleic acid sequence encoding a factor of the T-box family, thereby expressing a factor of the T-box family. In another embodiment, the invention relates to compositions comprising a vector which comprises a nucleic acid sequence encoding a factor of the T-box family and in another embodiment the composition comprising cell which expresses a factor of the T-box family, which includes *inter-alia* the brachyury.

0030 The term "cartilage " refers hereinabove to a specialized type of dense connective tissue consisting of cells embedded in a matrix. There are several kinds of cartilage. Translucent cartilage having a homogeneous matrix containing collagenous fibers is found in articular cartilage, in costal cartilages, in the septum of the nose, in larynx and trachea. Articular cartilage is hyaline cartilage covering the articular surfaces of bones. Costal cartilage connects the true ribs and the sternum. Fibrous cartilage contains collagen fibers. Yellow cartilage is a network of elastic fibers holding cartilage cells which is primarily found in the epiglottis, the external ear, and the auditory tube. Cartilage is tissue made up of extracellular matrix primarily comprised of the organic compounds collagen, hyaluronic acid (a proteoglycan), and chondrocyte cells, which are responsible for cartilage production. Collagen, hyaluronic acid and water entrapped within these organic matrix elements yield the unique elastic properties and strength of cartilage.

0031 As used herein, "hyaline cartilage" refers to the connective tissue covering the joint surface. By way of example only, hyaline cartilage includes, but is not limited to, articular cartilage, costal cartilage, and nose cartilage.

0032 As used herein, the term "enhancing cartilage repair" refers to healing and regeneration of cartilage injuries, tears, deformities or defects, and prophylactic use in preventing damage to cartilaginous tissue.

0033 As used herein, the term “inducing formation” refers to the use in cartilage renewal or regeneration so as to ameliorate conditions of cartilage, degeneration, depletion or damage such as might be caused by aging, genetic or infectious disease, accident or any other cause, in humans, livestock, domestic animals or any other animal species. In another embodiment the formation of a cartilage is required for cartilage development in livestock, domestic animals or any other animal species in order to achieve increased growth for commercial or any other purpose. In another embodiment the formation of a cartilage is required in plastic surgeries, such as without being limited facial reconstruction in order to obtain a stabilized shape.

0034 In one embodiment there is provided a recombinant vector comprising a nucleic acid sequence encoding a factor of the T-box family.

0035 The term “T-box family” defined as a family of transcription factors that share the T-box, a 200 amino acid DNA-binding domain. The T-box family have been identified in both vertebrates and in vertebrates and plays a key role in embryonic development.

0036 In another embodiment, the vector comprising a nucleic acid which encodes for *Brachyury*, or *T*, which refers hereinabove to the founder factor of the T-box family. It has been shown that *Brachyury* is highly expressed during gastrulation where it plays a decisive role in the generation of undifferentiated mesoderm and, thereafter, *Brachyury* expression is downregulated (reviewed in (Papaioannou and Silver, 1998; Smith, 1999).

0037 The immediate BMP2-dependent upregulation of FGFR3 in MSCs (C3H10T1/2) and the inherent capacity of this receptor to initiate chondrogenic development in these cells prompted a screen for FGFR3-regulated transcription factors. The chondrogenic potential of *Brachyury* after recombinant expression in wild-type C3H10T1/2 cells (see below) has been hypothesized after it was shown, by the use of a subtractive screening method, exemplified in Example 1 that, among the transcription factors tested, the T-box transcription factor *Brachyury* was upregulated in FGFR3-expressing C3H10T1/2 cells (see also Fig. 5 a).

0038 As used herein, the term "nucleic acid" refers to polynucleotides or to ologonucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA) or mimetics thereof. The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

0039 The terms "protein", "polypeptide" and "peptide" are used interchangably herein when referring to a gene product.

0040 The vector molecule can be any molecule capable of being delivered and maintained within the target cell or tissue such that the gene encoding the product of interest can be stably expressed. The vector molecule preferably utilized in the present invention is either a viral or retroviral vector molecule or a plasmid DNA non-viral molecule. This method preferably includes introducing the gene encoding the product into the cell of the mammalian connective tissue for a therapeutic or prophylactic use. Unlike previous pharmacological efforts, the methods of the present invention employ gene therapy to address the chronic debilitating effects of joint pathologies. The viral vectors used in the methods of the present invention can be selected form the group consisting of (a) a retroviral vector, such as MFG or pLJ; (b) an adeno-associated virus; (c) an adenovirus; and (d) a herpes virus, including but not limited to herepes simplex 1 or herpes simples 2 or (e)lentivirus. Alternatively, a non-viral vector, such as a DNA plasmid vector, can be used. Any DNA plasmid vector known to one of ordinary skill in the art capable of stable maintenance within the targeted cell or tissue upon delivery, regardless of the method of delivery utilized is within the scope of the present invention. Non-viral means for introducing the gene encoding for the product into the target cell are also within the scope of the present invention. Such non-viral means can be selected from the group consisting of (a) at least one liposome, (b) $Ca_3 (PO_4)_2$, (c) electroporation, (d) DEAE-dextran, and (e) injection of naked DNA.

0041 As will be appreciated by one skilled in the art, a fragment or derivative of a nucleic acid sequence or gene that encodes for a protein or peptide can still function in the same manner as the entire, wild type gene or sequence. Likewise, forms of nucleic acid sequences can have variations as compared with the wild type sequence, while the sequence still encodes a protein or peptide, or fragments thereof, that retain their wild type function despite these variations. Proteins, protein fragments, peptides, or derivatives also can experience deviations from the wild type from which still functioning in the same manner as the wild type form. Similarly, derivatives of the genes and products of interest used in the present invention will have the same biological effect on the host as the non-derivatized forms. Examples of such derivatives include but are not limited to dimerized or oligomerized forms of the genes or proteins, as wells as the genes or proteins modified by the addition of an immunoglobulin (Ig) group. Biologically active derivatives and fragments of the genes, DNA sequences, peptides and proteins of the present invention are therefore also within the scope of this invention. In addition, any nucleic acid which is cis acting and integrated upstream to an endogenous factor of the T-box family nucleic acid sequence is relevant to the present invention.

0042 The term "cis-acting" is used to describe a genetic region that serves as an attachment site for DNA-binding proteins (e.g. enhancers, operators and promoters) thereby affecting the activity of genes on the same chromosome.

0043 It was shown that Brachyury expression is upregulated by certain factor such as BMP2 and/or FGF3 (see fig.4 and 5). Thus, in another embodiment, the vector of the invention further comprising a nucleic acid which encodes to a protein which activated the BMP signaling pathway. In another embodiment, the protein which activated the BMP signaling pathway is a member of the BMP family. In another embodiment the BMP is a BMP2. In another embodiment the vector further comprising a nucleic acid for fibroblast growth factor namely FGF- 3.

0044 The term "protein which activates BMP mediated signaling pathway" is defined hereinabove as a protein that can activate the BMP receptors, or the signaling cascade down stream of the receptor to elicit BMP specific cellular response. Examples, without being limited are members of the BMP family, such as the BMP proteins BMP-1, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 and BMP-7, disclosed for instance in U.S. Pat. Nos. 5,108,922; 5,013,649; 5,116,738; 5,106,748; 5,187,076, and 5,141,905; BMP-8, disclosed in PCT

publication W091/18098; BMP-9, disclosed in PCT publication W093/00432; and BMP-10 or BMP-11, disclosed in co-pending patent applications, serial number 08/061,695 presently abandoned, a continuation-in-part of which has issued as U.S. Pat. No. 5,637,480, and 08/061,464 presently abandoned, a continuation-in-part of which has issued as U.S. Pat. No. 5,639,638 filed on May 12, 1993.

0045 In another embodiment, there is provided an engineered cell which expresses at least one factor of the T-box family.

0046 The term "engineered cell" is defined hereinabove to a cell or to a tissue which had been genetically modified and is expressing a factor of the T-box family or in another embodiment, increased amounts of the factor of the T-box family or in another embodiment express Brachury. The term "increased amount of the factor or the at least one factor of the T-box family refers hereinabove to at least 10 times more than normal.

0047 The cell of the invention can be any cell. In one embodiment, it is a mammalian cell. In another embodiment, it is a mesenchymal stem cell, in another embdoiment it is a progenitor cell, in another embodiment it is a cell derived from a cartilage. In another embodiment the cell can be derived from a fibroblast cell line, a mesenchymal cell line, a chondrocyte cell line, an osteoblast cell line, or an osteocyte cell line. The fibroblast cell line may be a human foreskin fibroblast cell line or NIH 3T3 cell line. In another embodiment the cell of the invention is a synovial cell or a synoviocyte. Synoviocytes are found in joint spaces adjacent to cartilage have an important role in cartilage metabolism. Synoviocytes produce metallo-proteinases, such as collagenases that are capable of breaking-down cartilage

0048 Stem cells are defined as cells which are not terminally differentiated, which can divide without limit, and divides to yield cells that are either stem cells or which irreversibly differentiate to yield a new type of cell. Those stem cells which give rise to a single type of cell are call unipotent cells; those which give rise to many cell types are called pluripotent cells. Chondro/osteoprogenitor cells, which are bipotent with the ability to differentiate into cartilage or bone, were isolated from bone marrow (for example, as described by Owen, J. Cell Sci. Suppl. 10, 63-76 (1988) and in U.S. Pat. No. 5,226,914 to Caplan, et al.).

0049 It is important to note that mesenchymal stem cells and progenitors can be isolated from different source tissues, skin, bone marrow, muscle, and liver. In addition any cell type with stem cell properties or demonstrating differentiation plasticity for example without limitation, SP cells from the source of bone marrow, muscle, spleen or any other tissue.

0050 Chondrogenic cells useful in the practice of the invention may be isolated from essentially any tissue containing chondrogenic cells. As used herein, the term "chondrogenic cell" is understood to mean any cell which, when exposed to appropriate stimuli, may differentiate into a cell capable of producing and secreting components characteristic of cartilage tissue. The chondrogenic cells may be isolated directly from pre-existing cartilage tissue, for example, hyaline cartilage, elastic cartilage, or fibrocartilage. Specifically, chondrogenic cells may be isolated from articular cartilage (from either weight-bearing or non-weight-bearing joints), costal cartilage, nasal cartilage, auricular cartilage, 30 tracheal cartilage, epiglottic cartilage, thyroid cartilage, arytenoid cartilage and cricoid cartilage. The cell from the cartilage can be derived from another animal, or another subject or in another embodiment, the cell of the cartilage or the bone can be derived from the subject in need.

0051 In another embodiment, the cell further express at least one protein which activates BMP mediated signaling pathway or a FGF protein.

0052 The expression of the at least one factor of the T-box family in combination with either and BMP or FGF or both in the cell can be due to the presence of two or more different vectors (trans vectors) or due to the expression of one vector which comprises two or more different nucleic acid sequences, which encode for the at least one member of the T-box family, the FGF and for at least one protein which activates the BMP mediated signaling pathway.

0053 In another embodiment the invention provides complex tissue engineering. This term refers to engineering a cell with different nucleic acid sequences, wherein each sequence encodes to a specific pathway of differentiation. As such, the cell of the invention can be engineered to differentiate to an osteoblast as well as to a chondrocyte.

0054 In another embodiment there is provided a composition comprising recombinant vector comprising a nucleic acid sequence encoding the a factor of the T-box family and a pharmaceutically acceptable carrier. It should be noted that the term "a nucleic acid sequence encoding the a factor of the T-box family" refers hereinabove to "at least one nucleic acid sequence encoding the at least one factor of the T-box family". Similarly the term "a cell" refers to "at least one cell".

0055 In another embodiment, there is provided a composition comprising at least one engineered cell, wherein said engineered cell expresses least one factor of the T-box family at least one protein and a pharmaceutically acceptable carrier.

0056 In another embodiment the composition can be a pharmaceutical composition.

0057 Compositions of the invention may further comprise additional proteins, such as additional factors. These compositions may be used to induce the formation or repair of cartilage tissue.

0058 The compositions of the invention may comprise, also BMP-12 or VL-1 (BMP-13), other therapeutically useful agents including MP52, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factors (TGF-α and TGF- β), and fibroblast growth factor-4 (FGF-4), parathyroid hormone (PTH), leukemia inhibitory factor (LIF/HILDA/DIA), insulin-like growth factors (IGF-I and IGF-II). Portions of these agents may also be used in compositions of the present invention. N another embodiment the composition comprises anti-inflammatory agents such as IL1 receptor antagonists, or IL4 or IL 10 agonists.

0059 In another embodiment, there is provided an implant device for transplantation in a subject in need comprising an engineered cell which expresses a factor of the T-box family and a pharmaceutically acceptable carrier. Cartilage implants are often used in reconstructive or plastic surgery such as rhinoplasty.

0060 The preparation and formulation of such pharmaceutically/physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity in factor of the T-box family due to high homology between species.

0061 Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with the compositions of the present invention.

0062 The therapeutic method includes administering the composition topically, systemically, or locally as an injectable and/or implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition, may desirably be encapsulated or injected in a viscous form for delivery to the site of tissue damage. Therapeutically useful agents other than the proteins which may also optionally be included in the composition as

described above, may alternatively or additionally, be administered simultaneously or sequentially with the composition in the methods of the invention. In addition, the compositions of the present invention may be used in conjunction with presently available treatments for cartilage injury such as cartilage allograft or autograft, in order to enhance or accelerate the healing potential of the or graft. For example, the, allograft or autograft may be soaked in the compositions of the present invention prior to implantation. It may also be possible to incorporate the protein or composition of the invention onto suture materials, for example, by freeze-drying.

0063 The compositions may include an appropriate matrix and/or sequestering agent as a carrier. For instance, the matrix may support the composition or provide a surface for cartilage-like tissue formation. The matrix may provide slow release of the protein and/or the appropriate environment for presentation thereof. The sequestering agent may be a substance which aids in ease of administration through injection or other means, or may slow the migration of protein from the site of application.

0064 The choice of a carrier material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined. Preferred matrices include collagen-based materials, including sponges, such as Helistat.RTM. (Integra LifeSciences, Plainsboro, N.J.), or collagen in an injectable form, as well as sequestering agents, which may be biodegradable, for example hyalouronic acid derived. Biodegradable materials, such as cellulose films, or surgical meshes, may also serve as matrices. Such materials could be sutured into an injury site, or wrapped around the tendon/ligament.

0065 Another preferred class of carrier are polymeric matrices, including polymers of poly(lactic acid), poly(glycolic acid) and copolymers of lactic acid and glycolic acid. These matrices may be in the form of a sponge, or in the form of porous particles, and may also include a sequestering agent. Suitable polymer matrices are described, for example, in W093/00050, the disclosure of which is incorporated herein by reference.

0066 Preferred families of sequestering agents include blood, fibrin clot and/or cellulosic materials such as alkylcelluloses (including hydroxyalkylcelluloses), including

methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, and carboxymethylcellulose, the most preferred being cationic salts of carboxymethylcellulose (CMC). Other preferred sequestering agents include hyaluronic acid, sodium alginate, poly(ethylene glycol), polyoxyethylene oxide, carboxyvinyl polymer and poly(vinyl alcohol). The amount of sequestering agent useful herein is 0.5-20 wt %, preferably 1-10 wt % based on total formulation weight, which represents the amount necessary to prevent desorbtion of the protein from the polymer matrix and to provide appropriate handling of the composition, yet not so much that the progenitor cells are prevented from infiltrating the matrix, thereby providing the protein the opportunity to assist the activity of the progenitor cells.

0067 Additional optional components useful in the practice of the subject application include, e.g. cryogenic protectors such as mannitol, sucrose, lactose, glucose, or glycine (to protect the protein from degradation during lyophilization), antimicrobial preservatives such as methyl and propyl parabens and benzyl alcohol; antioxidants such as EDTA, citrate and BHT (butylated hydroxytoluene); and surfactants sifdch as poly(sorbates) and poly(oxyethylenes); etc.

0068 As described above, the compositions and the devices of the invention may be employed in methods for enhancing cartilage repair or for inducing cartilage formation. These methods, according to the invention, entail administering to a patient needing such tissue repair, a cell expresses at least one factor of the T-box family or in another embodiment a composition comprising an effective amount of vector comprising a nucleic acid encoding a factor of the T-box family.

0069 In another embodiment, as described before, the composition or the cell may comprise also a vector comprising a nucleic acid encoding FGF and/or a factor of the BMP family.

0070 Preferably the DNA molecule or protein may be injected directly into cartilage tissue such as without limitation nasal cartilage, articular cartilage etc. Therefore, the compounds of the invention may be utilised as a therapeutic agent in regard to treatment of cartilage or bone damage caused by disease or aging or by physical stress such as occurs through injury or repetitive strain, e.g. "tennis elbow" and similar complaints. The therapeutic agent of the invention may also be utilised as part of a suitable drug delivery system to a particular tissue that may be targeted.

0071 Other therapeutic applications for the compounds of the invention may include the following: 1. Use in cartilage and/or bone renewal, regeneration or repair so as to ameliorate conditions of cartilage and/or bone breakage, degeneration, depletion or damage such as might be caused by aging, genetic or infectious disease, wear and tear, physical stress (for example, in athletes or manual labourers), accident or any other cause, in humans, livestock, domestic animals or any other animal species; 2. Stimulation of skeletal development in livestock, domestic animals or any other animal species in order to achieve increased growth for commercial or any other purpose; 3. Treatment of neoplasia or hyperplasia of bone or cartilage, in humans, livestock, domestic animals or any other animal species; 4. Suppression of growth of skeletal components in livestock, domestic animals or any other animal species in order to achieve decreased growth for commercial or any other purposes e.g. by the use of antisense molecules to the factor of the T-box family; and 5. Alteration of the quality or quantity of cartilage and/or bone for any other purpose in any animal species including humans.

Thus, according to clauses 4 and 5 the invention can be serve also for suppressing cartilage formation, by the use of an antagonist to Brachyury or to other factors of the T-box family. The antagonistic effect of dominant negative Brachyury is exemplified in Example 3. The term "antagonist" refers to a molecule which, when bound to the epitope, decreases the amount or the duration of the effect of the biological or immunological activity of epitope. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, antisense or any other molecules which decrease the effect of Brachyury or to other factors of the T-box family on cartilage formation. Such a treatment of suppression is relevant in the treatment of malignancy of the cartilage, for example without limitation in chondroma and chondrasarcoma.In another embodiment the antagonist is a dominant negative factor of the T-box family. In another embodiment the antagonist is a dominant negative Brachyury.

The term “dominant negative Brachyury refers hereinabove to Brachyury DNA binding domain (T-box, aa 1-229) without the associated regulatory domains (aa 230-436).

0072 In another embodiment the invention provides a method of inducing chondrocyte differentiation comprising the step of administering of a recombinant vector which comprises a nucleic acid encoding a factor of the T-box family, thereby inducing chondrocyte formation. Example 2 and figure 2 clearly demonstrates that forced expression of the T-box factor Brachyury leads to chondrgenic development in C3H10T1/2 mesenchymal stem cells. Please

note that C3H10T1/2 mesenchymal stem cells line. C3H10T1/2 stem cell line resembles human MSCs by many features including differentiation multipotentiality, high proliferation capacity and similar response to growth factors and cytokines such as BMPs.

0073 Example 3 further strengthen the relation between Brachyury and chondrgenic development, by demonstrating that dominant negative Brachyury interferes with BMP2 dependent chondrogenic development in mesenchymal cells.

0074 In another embodiment the invention relates to a method for the production of transplantable cartilage matrix, the method comprising the steps of: obtaining a cell; transfecting said cell with a recombinant vector comprising a nucleic acid sequence encoding a factor of the T-box family, so as to obtain an engineered cell which expresses a factor of the T-box family; and culturing said cell with the cell-associated matrix for a time effective for allowing formation of a transplantable cartilage matrix. The above method will enable the production of a cartilage matrix which will be transplanted to a subject in need when required.

0075 In another embodiment, there is provided a method of treating a subject by ex-vivo implantation of at least one cell comprising the following steps: obtaining at least one cell from the subject; transfecting the cell with a nucleic acid which encodes at least one factor of the T-box family, so as to obtain an cell which express at least one factor of the T-box family activated cell; and administering said activated cell to the subject.

0076 Optionally, the enriched stem cells are then expanded ex vivo by culturing them in the presence of agents that stimulate proliferation of stem cells. The culturing atep can be for example in a bioreactor which enables three dimensional growth of the cells. The enriched and optionally expanded stem cells are then infected with a vector, that expresses the at least one factor of the T-box family gene. Optionally, the vector may also carry an expressed selectable marker, in which case successfully transduced cells may be selected for the presence of the selectable marker. The transduced and optionally selected stem cells are then returned to the patient defective connective tissue and allowed to engraft themselves into the bone marrow.

0077 One ex vivo method of enhancing repair and/or inducing formation disclosed throughout this specification comprises initially generating a recombinant viral or plasmid vector which contains a DNA sequence encoding a protein or biologically active fragment thereof. This recombinant vector is then used to infect or transfect a population of in vitro cultured connective tissue cells, resulting in a population of connective cells containing the

vector. These connective tissue cells are then transplanted to a target joint space of a mammalian host, effecting subsequent expression of the protein or protein fragment within the joint space. Expression of this DNA sequence of interest is useful in substantially reducing at least one deleterious joint pathology associated with a connective tissue disorder.

0078 It will be understood by the artisan of ordinary skill that the source of cells for treating a human patient is the patient's own connective tissue cells, such as autologous fibroblast cells. In another embodiment the source of cells can be allogenic cells which were treated so as to reduce immune response.

0079 As used herein, a "promoter" can be any sequence of DNA that is active, and controls transcription in an eucaryotic cell. The promoter may be active in either or both eucaryotic and procaryotic cells. In another embodiment , the promoter is active in mammalian cells. The promoter may be constitutively expressed or inducible. In another embodiment , the promoter is inducible. In another embodiment , the promoter is inducible by an external stimulus. In another embodiment , the promoter is inducible by hormones or metals. Still more in another embodiment , the promoter is inducible by heavy metals. In another embodiment , the promoter is a metallothionein gene promoter. In another embodiment the promoter is inducible by antibiotics such as tetracycline. In another embodiment the promoter is inducible by a tissue specific promoter. Likewise, "enhancer elements", which also control transcription, can be inserted into the DNA vector construct, and used with the construct of the present invention to enhance the expression of the gene of interest.

0080 In another embodiment there provided ex vivo and in vivo techniques for delivery of a DNA sequence of interest to the connective tissue cells of the mammalian host. The ex vivo technique involves culture of target connective tissue cells, in vitro transfection of the DNA sequence, DNA vector or other delivery vehicle of interest into the connective tissue cells, followed by transplantation of the modified connective tissue cells to the target joint of the mammalian host, so as to effect in vivo expression of the gene product of interest.

0081 Alternatively, an allograft (e.g., cartilage grown in vitro from cartilage tissue removed from the patient) may be implanted by attaching a periosteum membrane (harvested, e.g., from the patient's tibia), to the bone surface and injecting the allograft beneath the membrane.

0082 As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

0083 Alternatively, the gene encoding the product of interest can be associated with liposomes and injected directly into the host, such as in the area of the joint, where the liposomes fuse with target cells, resulting in an in vivo gene transfer to the connective tissue. In another embodiment, the gene encoding the product of interest is introduced into the area of the joint as naked DNA. The naked DNA enters the target cells, resulting in an in vivo gene transfer to the cells.

0084 The dosage of the treatment, which is the amount of the cells which express the at least one factor of the T-box family or in another embodiment the amount of the composition or the device which contain the vector comprising the nucleic acid encoding the same in the in vivo and in the ex-vivo treatment the dosage regimen will be determined by the attending physician considering various factors which modify the action of the composition, e.g., amount of bone or cartilage tissue desired to be formed, the site of the bone or cartilage damage, the condition of the damaged cartilage or bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of additional proteins in the composition. The addition of other known growth factors, such as IGF-I (insulin like growth factor I), to the final composition, may also affect the dosage.

0085 Progress can be monitored by periodic assessment of cartilage formation, and/or repair. The progress can be monitored by methods known in the art, for example, X-rays (CT), ultra-sound, MRI, arthroscopy and histomorphometric determinations.

0086 In another embodiment, as is examplified in Example 1 the invention provides a method of screening candidate nucleic acid sequence which is involved in the early stages of cartilage development, said method comprising the step of: obtaining a cell; transfecting said cell with a vector comprising a nucleic acid sequence encoding to FGFR3; obtaining mRNA from said cell; synthesizing cDNA from said mRNA; amplifying said cDNA-hybrid, so as to obtain an amplified product; detecting said amplified product; and comparing said amplified products from said sample to amplified products derived from known samples thereby identifying candidate nucleic acid sequence which is involved in the early stages of cartilage development.

0087 The term “involved in the early stages of cartilage development” refers hereinabove to any gene which is either upregulated on downregulated during the stage of differentiation into a cartilage cell. Such genes will enable development of drugs which will ether enhance or suppress cartilage formation or repair.

0088 The step of “synthesizing” refer to step of building cDNA complementary to the mRNA template. As refer hereinabove and in the claims section, the step of “amplifying” refer to the selective replication of a cDNA in greater number than usual. As refer herein above and in the claims section, the step of “separating” refer to the step of separation of the products using for example, gel electrophoresis. As refer hereinabove and in the claims section, the step of “detecting” refer to the step of noticing, which is done, for example by visualization of the amplified product’s bands. As refer hereinabove and in the claims section, the step of “comparing” refers to the step of searching for differences between the amplified products derived from the at least two samples. The term "RNA" refers to an oligonucleic in which the sugar is ribose, as opposed to deoxyribose in DNA. RNA is intended to include any nucleic acid, which can be entrapped by ribosomes and translated into protein. The term “mRNA” refers to messenger RNA.

RNA can be extracted from cells or tissues according to methods known in the art. In a preferred embodiment, RNA can be extracted from monolayers of mammalian cells grown in tissue culture, cells in suspension or from mammalilan tissue. RNA can be extracted from such sources by, e.g., treating the cells with proteinase K in the presence of SDS. In another embodiment, RNA is extracted by organic solvents. In yet another embodiment, RNA is extracted by differential precipitation to separate high molecular weight RNA from other nucleic acids. RNA can also be extracted from a specific cellular compartment, e.g., nucleus or the cytoplasm. In such methods, the nucleus is either isolated for purification of RNA therefrom, or the nucleus is discarded for purification of cytoplasmic RNA. Further details regarding these and other RNA extraction protocols are set forth, e.g., in Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989).

EXAMPLES

EXPERIMENTAL PROCEDURES

0089 **DNA constructs and Transient Transfections**

0090 For the assessment of the transcriptional activity a dimer of the double-stranded oligonucleotide of the *Brachyury* binding element (BBE) AATTTCACACCTAGGTGTGAAATT (Kispert et al., 1995) was incorporated in the *Bam*HI site before the HSV thymidine kinase minimal promoter fused to the cloramphenicol acetyltransferase (CAT)-reporter of pBLCAT5 (Boshart et al., 1992) to give reporter plasmid pBBE-CAT5. 20 h before transfection, human embryonic kidney HEK293T cells were plated at a density of 1 x 10^4 /cm^2 in 6-well plates and allowed to grow under normal culture conditions. For co-transfection experiments, 250 ng per well of Brachyury expression vector and 250, 500 or 750 ng of the expression vector encoding dn*Brachyury*. Empty vector was added to adjust the amount of expression plasmids at 1ug/ml. 260 ng of BBE-CAT reporter (pBBE-CAT5) was added in the presence of 140 ng of RSV-lacZ vector using the DOSPER procedure (see below). Cells were allowed to incubate for 48 h. Then, cells were collected and b-galactosidase assays were performed with the chemiluminescent b-gal reporter gene assay (Roche Diagnostics, Mannheim, Germany) and CAT-assays were carried out with the CAT ELISA kit (Roche Diagnostics, Mannheim, Germany). b-gal assay results were used to normalize the CAT assay results for transfection efficiency. All DNA transfection experiments were repeated at least three times in triplicate.

0091 **Cell Culture and permanent Transfections**

0092 Human embryonic kidney cells HEK293T and murine C3H10T1/2 progenitor cells were routinely cultured in tissue culture flasks in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 0.2 mM L-glutamine, and antibiotics (50 units/ml penicillin, 50 mg/ml streptomycin). Cells were transfected using DOSPER according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). C3H10T1/2 cells which recombinantlly express BMP2 (C3H10T1/2-BMP2) cells were obtained by co-transfection with pSV2pac followed by selection with puromycin (2.5 ug/ml). FGFR3,

Brachyury and T-box domain were PCR-amplified and cloned into expression vectors pMT7T3 and pMT7T3-pgk vectors which are under the control of the LTR of the myeloproliferative virus or of the murine phosphoglycerate kinase promoter-1, respectively (Ahrens et al., 1993). The integrity of the constructs was confirmed by sequencing. HA-tags were carboxyterminally added to full-length *Brachyury* and *Brachyury's* T-box domain by PCR with primers encoding the respective peptide sequence. Stable expression of the DNA binding T-box domain (aa 1-229) and of the dominant-negative human FGFR3 without the cytoplasmatic tyrosine kinase domains (aa 1-414) in the C3H10T1/2-BMP2 background was done by co-transfection with pAG60, conferring resistance to G418 (750 ug/ml). Individual clones were picked, propagated, and tested for recombinant FGFR3, dnFGFR3, *Brachyury* or T-box domain (dn*Brachyury*) expression by RT-PCR (see below). Selected cell clones were subcultivated in the presence of puromycine or puromycine/G418 and the selective pressure was maintained during subsequent manipulations. C3H10T1/2 cells were cultured in DMEM containing 10% fetal bovine serum. The features of C3H10T1/2-BMP2 cells have been described (Ahrens et al., 1993; Hollnagel et al., 1997; Bächner et al., 1998). For the assessment of *in vitro* osteo- /chondrogenic development, cells were plated at a density of 5-7.5 x10^3 cells/cm^2. After reaching confluence (arbitrarily termed day 0) ascorbic acid (50 ug/ml) and 10 mM b-glycerophosphate were added as specified by Owen et al., 1990 (1990).

0093 **BMP2 inductions**

0094 For BMP2-stimulation studies, C3H10T1/2 cells were plated at a density of 1x10^4 per cm^2 in a 9-cm culture dish. After 48 h cells were washed 3x with PBS and then cells were starved for 24 h in DMEM without serum. Before induction the medium was replaced with fresh DMEM without serum. Cells were then treated for the indicated times using recombinant BMP2 from *E. coli* (50 ng/ml). Cycloheximide (50 ug/ml) treatment started 30 min prior to the addition of BMP2.

0095 **RNA preparation and RT-PCR**

0096 Total cellular RNAs were prepared by TriReagent^{LS} according to the manufacturer's protocol (Molecular Research Center Inc.). Five ug of total RNA was reverse transcribed and cDNA aliquots were subjected to PCR. RT-PCR was normalized by the transcriptional levels of HPRT. The HPRT-specific 5' and 3' primers were GCTGGTGAAAAGGACCTCT and

AAGTAGATGGCCACAGGACT, respectively. The following 5' and 3' primers were used to evaluate osteo/chondrogenic differentiation: collagen 1a1: GCCCTGCCTGCTTCGTG, CGTAAGTTGGAATGGTTTTT; collagen 2a1: CCTGTCTGCTTCTTGTAAAAC, AGCATCTGTAGGGGTCTTCT; osteocalcin: GCAGACCTAGCAGACACCAT, GAGCTGCTGTGACATCCATAC; PTH/PTHrP-receptor: GTTGCCATCATATACTGTTTCTGC, GGCTTCTTGGTCCATCTGTCC; FGFR3: CCTGCGCAGTCCCCCAAAGAAG; CTGCAGGCATCAAAGGAGTAGT; FGFR2: TTGGAGGATGGGCCGGTGTGGTG, GCGCTTCATCTGCCTGGTCTTG. The primer pairs for *Brachyury* and *Sox9* have been described in (Johansson and Wiles, 1995) and (Zehentner et al., 1999), respectively. Vector-borne transcripts for *Brachyury* were evaluated with nested primers sets with either vector specific 5'- or 3'-primers: TTAGTCTTTTTGTCTTTTATTTCA; GATCGAAGCTCAATTAACCCTCAC.

0097 **Western blotting**

0098 Recombinant cells from petri dishes (13.6 cm diameter) were harvested at different time points before (day B2), at (day 0) and after (days 2, 4, 7) confluence. Lysis was in RIPA buffer (1% (v/v) nonidet P-40, 0.1% SDS (w/v), 0.5% sodium deoxycholate in PBS, containing 100 ug/ml PMSF, 2 ug/ml aprotinin, and 1 mM Na_3VO_4). Lysates were centrifuged (30 min, 10.000 g, 4C) and the supernatants were stored at -70C until analysis. Protein concentration of the lysates was determined using coomassie brilliant blue. Protein was precipitated with ethanol, resuspended in reducing (containing DTT) or non-reducing sample buffer and subjected to SDS-gel electrophoresis in 12.5%T polyacrylamide gels (20ug/lane). Proteins were transferred to nitrocellulose membranes by semidry-blotting. Protein transfer was checked by staining of the membranes with Ponceau S. After blocking, membranes were incubated incubated overnight at 4 C with a polyclonal antibody to the HA-tag (SC-805, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1: 200 in blocking solution. FGFR3 and FGFR2 antibodies were from Santa Cruz Biotechnology (#SC-123, #SC-122; Santa Cruz, CA). The secondary antibody (Dianova, Hamburg) was applied at 1: 5000 in blocking solution for 2 h at room temperature. Color development was performed with 4-chloro-1-naphthol and H_2O_2.

0099 **Histological Methods and Verification of Cellular Phenotypes**

00100 Osteoblasts exhibit stellate morphology displaying high levels of alkaline phosphatase, which was visualized by cellular staining with SIGMA FAST BCIP/NBT (Sigma, St. Louis, MO). Proteoglycan secreting chondrocytes were identified by staining with Alcian Blue at pH 2.5 and staining with Safranin O (Sigma, St. Louis, MO). For collagen-immunohistochemistry cells were washed with PBS and fixed with methanol for 15 min at -20 C by methanol. Primary antibodies were diluted with 1% goat serum in PBS. Monoclonal anti-collagen II antibodies (Quartett Immunodiagnostika, Berlin, Germany, # 031502101) were diluted 1:50 and monoclonal anti-collagen X antibodies (Quartett Immunodiagnostika, Berlin, Germany, # 031501005) 1:10, respectively. Incubation was for 1 hour at room temperature followed by staining with Zymed HistoStain SP kit (Zymed Laboratories Inc., San Francisco, CA) applying the manufacturer's protocol. A positive signal is indicated by a red color precipitate of AEC (aminnoethylcarbazole).

00101 ***In Vivo Transplantation***

00102 Before *in vivo* transplantation, aliquots of 2-3x10^6 cells were mounted on individual type I collagen sponges (Colastat[7] #CP-3n, Vitaphore Corp., 2 x 2 x 4mm.) and transplanted into the abdominal muscle of female nude mice (4-8 weeks old). Before transplantation animals were anaesthetized with ketamine-xylazine mixture 30ul/per mouse i.p. and injected i.p. with 5mg/mouse of Cefamzolin (Cefamezin[7], TEVA). Skin was swabbed with chlorhexidine gluconate 0.5%, cut in the middle abdominal area, an intramuscular pocket was formed in a rectal abdominal muscle and filled with the collagen sponge containing cells. Skin was sutured with surgical clips. For the detection of engrafted C3H10T1/2 cells the mice were sacrificed 10 days and at 20 days after transplantation. Operated transplants were fixed in 4% paraformaldehyde cryoprotected with 5% sucrose overnight, embedded, and frozen. Sections were prepared with a cryostat (Bright, model OTF) and stained with H&E, Alcian Blue and Safranin O.

00103 **RNA-In Situ-Hybridization**

00104 Embryos were isolated from pregnant NMRI mice at day 18.5 post conceptionem (dpc). The embryos were fixed overnight with 4% paraformaldehyde in PBS at 4C. 10 um cryosections were mounted on aminopropyltriethoxysilane coated slides and non-radioactive

RNA-*in situ*-hybridizations were done as described (Bächner et al., 1998) and by following the instructions of the manufacturer (Roche, Mannheim). In short: For hybridization sense- and antisense RNA probes from a 1.8 kb murine *Brachyury* cDNA was used. For the generation of collagen 1a1 or collagen 2a1 the vector pMT7T3 was used harbouring specific probes (Metsäranta et al., 1991). Hybridization was performed with 0.5 - 2 ug denatured riboprobe/ml) over night at 65C in a humid chamber. For digoxygenin (DIG)-detection slides were blocked in 5 x SSC, 0.1% Triton, 20% FCS for 30 minutes following two washes with DIG-buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.6) for 10 minutes. Slides were incubated in anti-DIG-alkaline phosphatase coupled antibodies diluted 1:500 in DIG-buffer 1 over night in a humid chamber. Slides were washes with 0.1% Triton in DIG-buffer 1 for 2 hours with several changes of the washing solution and equilibrated in DIG-buffer 2 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$). Detection was performed using BM-purple substrate (Roche, Mannheim) in DIG-buffer 2 with 1mM Levamisole for 1 - 6 hours depending on the probe. Reaction was stopped in TE-buffer and slides were incubated in 3% paraformaldehyde in PBS for 3 minutes, in 0.1 M glycine in PBS for 3 minutes and washed three times in PBS for 3 minutes. Slides were counterstained with 0.5% methylenegreen in PBS for 1 minute, dehydrated in graded alcohol series, air dried and mounted with Eukitt.

EXPERIMENTAL RESULTS

EXAMPLE 1

00105 **BMP2-dependent Chondrogenic Development in C3H10T1/2 MSCs Involves FGF-Receptor 3**

00106 During a substractive screen for BMP-regulated genes in recombinant BMP2-expressing C3H10T1/2 (C3H10T1/2-BMP2) cells upregulation of the Fibroblast Growth Factor Receptors 3 and 2 (FGFR3, FGFR2) was noted at both the transcriptional and protein levels (Fig. 1 a, c, respectively). These two receptor types exhibit different induction kinetics. FGFR3 is upregulated during early stages of cultivation in the stable C3H10T1/2-BMP2 line while FGFR2 shows a delayed response (Fig. 1a, c). The fast upregulation of FGFR3 seems to be due to an immediate response to BMP2 since exogenously-added BMP2 mediated FGFR3 transcription in wild-type C3H10T1/2 cells in the presence of cycloheximide (Fig. 1 b). In

contrast to FGFR3 and FGFR2 is FGFR1 constitutivelly expressed in wild type and C3H10T1/2-BMP2 cells (Fig. 1a) while FGFR4 does not show any significant rates of expression (data not shown). Since FGFs and their receptors are crucial modulators of chondrogenic development, an assessment of whether the immediate BMP2-dependent upregulation of FGFR3 in C3H10T1/2 is involved in the onset of chondrogenic differentiation was conducted. Indeed the results demonstrated that forced expression of the wild-type FGFR3 ($FGFR3^{WT}$) was sufficient for the development of morphologically distinct chondrocytes in C3H10T1/2-$FGFR3^{WT}$ cells (Fig. 1 d, e). Moreover, the constitutively active mutant FGFR3 (*Ach*, G380R) possesses the same capacity (data not shown). The forced expression of $FGFR3^{WT}$ in MSCs stimulates MAPK signaling in these cells as documented by enhanced levels of ERK1 and ERK2 phosphorylation (right panels in Fig. 1d), leads to the development of histologically distinct chondrocytes and induces or increases expression of chondrogenic marker genes such as collagen 2a1, the PTH/PTHrP receptor and transcription factor *Sox9* (Fig.1 e). In C3H10T1/2 cells *Sox9* is already expressed at substantial levels that are further upregulated by BMP2- and FGFR3 which is consistent with recent observations (Fig. 1e) (Zehentner et al., 1999; Murakami et al., 2000).

00107 The immediate BMP2-dependent upregulation of FGFR3 in MSCs (C3H10T1/2) and the inherent capacity of this receptor to initiate chondrogenic development in these cells prompted a screen for FGFR3-regulated transcription factors. It was observed that among the transcription factors tested, the T-box transcription factor *Brachyury* was upregulated in FGFR3-expressing C3H10T1/2 cells (see also Fig. 5 a). Thereupon, the chondrogenic potential that *Brachyury* possesses after recombinant expression in wild-type C3H10T1/2 cells (see below) has been hypothesized.

EXAMPLE 2

00108 **Forced Expression of the T-box Factor Brachyury Leads to Chondrogenic Development in C3H10T1/2 Mesenchymal Stem Cells**

00109 *Brachyury* has originally been described as the first member of a family of transcription factors that harbors a *T*-box as the DNA-binding domain. In order to assess whether the FGFR3-dependent upregulation of the T-box factor *Brachyury* in C3H10T1/2 might play a role in chondrogenesis *Brachyury* cDNA was expressed under the control of the

murine phosphoglycerate kinase-1 (PGK-1) in mesenchymal stem cell line C3H10T1/2 to allow moderate expression levels of *Brachyury* (C3H10T1/2-*Brachyury*). The recombinant expression of *Brachyury* cDNA under the control of the murine phosphoglycerate kinase-1 (PGK-1) in MSCs (Fig. 2 a) gave rise to efficient chondrogenic differentiation resulting in alkaline phosphatase positive cells (beginning at day 4) and Alcian Blue positive chondrocyte-like cells (at day 10 post-confluence; Fig. 2 b). Three individual C3H10T1/2 clones were investigated in regard to their chondrogenic potential and gave similar results. Immunohistochemistry confirms the presence of the chondrocyte-specific collagen 2 but not of collagen X which is typical for late stages of chondrocytic differentiation (hypertophic chondrocytes) (Fig. 2b, left panel). Major marker genes of chondrogenic and, also, of osteogenic development show a transient (collagen 2a1, PTH/PTHrP-receptor) or permanent upregulation (osteocalcin gene and the chondrogenic transcription factor *Sox9*) in C3H10T1/2-*Brachyury* in comparison with C3H10T1/2 cells which were stably transfected with an empty expression vector (Fig. 2c). Although, the induction of the osteocalcin gene indicates an osteogenic potential for C3H10T1/2-*Brachyury*, ectopic transplantation of these cells in murine intramuscular sites results exclusively in the massive formation of proliferating chondrocytes and cartilage (Fig. 2d). These ectopic transplantations have been performed three times and in all cases these transplants developed chondrocytes and cartilage. After both 10 and 20 days transplants exhibit a histological presence of proteoglycans (Alcian Blue, Safranin O) while bony elements or mineralized particles are not observed (Fig. 2 d). After 20 days the ectopic implants show areas of extensive extracellular matrix production as visualized by histological analyses (Fig. 2 d). The use of stronger viral promoters such as the LTR of the myeloproliferative virus (MATERIALS and METHODS) resulted in increased cellular proliferation without the apparent formation of histologically distinct mesenchymal cell types (not shown).

EXAMPLE 3

00110 **Dominant-negative Brachyury Interferes with BMP2-dependent Chondrogenic Development in MSCs.**

00111 It was expected that *Brachyury*'s DNA-binding domain (*T*-box, aa 1-229) without the associated regulatory domains (aa 230-436) should dominant-negatively (dn) interfere

with endogenous *Brachyury*-mediated events in C3H10T1/2-BMP2 cells. A partial nuclear localization signal (NLS) which has been attributed to the T-box domain should allow a substantial nuclear accumulation (Kispert et al., 1995). The dominant-negative nature of the T-box domain was confirmed in DNA co-transfection assays performed in HEK293 T cells. This particular cell lines was used because expression levels are in general considerably higher in these cells than in C3H10T1/2. This cell line does not express *Brachyury* (data not shown). Exogenous *Brachyury* transactivated a construct containing two copies of the consensus *Brachyury* binding element (BBE) oligonucleotide fused to a minimal HSV thymidine kinase (TK)-minimal promoter-CAT chimeric gene, pBBE(2x)-CAT5 (Fig. 3a). Indeed, co-transfection of pBBE(2x)-CAT5 with a recombinant *Brachyury*-expressing vector resulted in a 25-fold activation, whereas an empty expression vector had no effect (Fig.3a). Co-transfection of full-length *Brachyury* (*Brachyury* wt) with increasing amounts of an expression vector expressing the T-box domain (dn*Brachyury*) (1:1, 1:2, 1:3) led to a clear decrease in CAT (7-fold). Exogenous dnBrachyury alone transactivated pBBE(2x)-CAT5 (BBE) only 3-fold.

00112 The forced expression of the HA-tagged *T*-box domain (dn*Brachyury*) is observed throughout *in vitro* cultivation (Fig. 3b) and strongly interfered with the BMP2-mediated formation of alkaline phosphatase positive osteoblast-like and Alcian Blue chondrocyte-like cells *in vitro* (Fig. 3 e). *In vivo,* in ectopic transplantations of C3H10T1/2-BMP2 in intramuscular sites, dn*Brachyury* allowed the development of connective tissue only (Fig. 3 e). In addition, the chondrocyte-specific collagen 2a1 mRNA levels are more sensitive to the presence of dn*Brachyury* than mRNA levels of the distinct osteogenic marker osteocalcin. The latter is hardly affected, consistent with the idea that *Brachyury* possesses a predominant chondrogenic capacity in this particular cell type. Interestingly, the BMP2-mediated transcriptional upregulation of FGFR3 in C3H10T1/2 is not obstructed by dn*Brachyury* indicating that the immediate BMP2-mediated FGFR3 induction is independent of *Brachyury* or other T-Box factors (Fig. 3c, d). However, FGFR2-expression which exhibits a delayed response in C3H10T1/2-BMP2 cells (Figs. 1 and 3) displays a high sensitivity to dn*Brachyury*. BMP-mediated FGFR2 expression is almost completely suppressed by the dominant-negative acting T-box domain (Fig. 3 c, d). This may indicate that that the presence of FGFR2 seems necessary for the osteo- /chondrogenic differentiation in this mesenchymal progenitor line (Fig. 3 c, d).

00113 Furthermore, this suggests a hierarchy of FGFR-mediated signaling for chondrogenic development. FGFR3-dependent signaling is induced at first by BMP2 and as a consequence, FGFR2 mediated signaling becomes active. Such a model is proposed in Fig. 7. This model predicts that a forced expression of dominant-negative FGFR3 would interfere with BMP2-mediated chondrogenesis and with FGFR2 and *Brachyury* expression. Indeed, an FGFR3-variant without the cytoplasmatic tyrosine-kinase domains downregulates BMP2-dependent mRNA expression levels of FGFR2 and *Brachyury* (Fig. 4b) and interferes with the histological manifestation of alkaline phosphatase or Alcian Blue positive chondrocyte-like cells (Fig. 4a).

EXAMPLE 4

00114 **FGFR3 and the T-box Factor Brachyury Are Involved in an Autoregulatory Loop for Chondrogenic Development in C3H10T1/2 Progenitors**

00115 During amphibian gastrulation, mesodermal *Brachyury* is involved in an autoregulatory loop with FGF that is present in the embryo (1998). In C3H10T1/2 cells several FGF genes tested (FGF2, 4, and 9) were not *Brachyury*- or FGFR3-regulated (data not shown) and, therefore, are unlikely members of such a loop. However, a loop seems to exist between FGFR3 and *Brachyury* since forced expression of either one lead to the induction of the other one in C3H10T1/2 (Fig. 5a). These experiments indicate that after BMP2-mediated initiation of the chondrogenic lineage, the chondrogenic differentiation may advance for some time in a BMP2-independent fashion maintained by the autoregulatory loop between FGFRs and FGF-regulated transcription factors such as the T-box factor *Brachyury*.

00116 In a preceding study it was shown that BMP-mediated R-Smad signaling alone is not sufficient for cartilage development in C3H10T1/2 cells. Thereby, forced expression of Smad1 or the biologically active Smad1-MH2 domain is able to mimic BMP2-mediated onset of osteogenic differentiation (Takeuchi et al., 2000). However, in contrast to osteogenic marker genes such as the osteocalcin gene, Smad1-MH2 domain-signaling is not sufficient to mimic BMP2-dependent FGFR3- and the concomitant *Brachyury*-gene induction (Fig. 5b). Other BMP-activated R-Smads such as Smad5 and Smad8 are also unable to mediate or to mimic BMP2-dependent FGFR3-induction in C3H10T1/2 cells (data not shown) indicating

R-Smad-MH2-independent pathways for FGFR3 induction or, alternatively, cooperative activities of R-Smads with other transcription factors (Mazars et al., 2000).

EXAMPLE 5

00117 **The T-box Factor Brachyury Is Expressed in Maturing Cartilage During Murine Embryonic Development**

00118 The rule of *Brachyury* skeletogenesis *in vivo* was also assessed. *Brachyury* is expressed at high levels early in vertebrate embryonic development and is involved in gastrulation and in the dose-dependent determination of mesodermal cell fates (see INTRODUCTION, DISCUSSION). After gastrulation, *Brachyury*-expression is downregulated and persists in the notochord to the end of embryogenesis (Kispert and Herrmann, 1994). Comparative mRNA expression analysis of murine *Brachyury* (Bra), collagen 1a1 (col 1a1) and collagen 2a1 (col 2a1) in skeletal development (18.5 dpc) indicates that *Brachyury* is expressed at significant levels in cartilage forming cells of the intervertebral disks and in limb bud development (Fig. 6). Expression of *Brachyury* is enhanced in intervertebral disc development in the nucleus pulposus in 18.5 dpc mouse embryos (Fig. 6A, a, d) confirming earlier reports (Wilkinson et al., 1990). Collagen 1a1 is expressed in the outer annulus (arrowheads in Fig 6A b, e), and collagen 2a1 in the cartilage primordium of the vertebrae (Fig. 6A, c, f). In transversal sections made at the level of the upper lumbar vertebra, expression of *Brachyury* is in addition detectable in distinct chondrogenic cells of the neural arch (Fig. 6A, h) whereas collagen 1a1 expression is maintained in the outer annulus (Fig. 6A, i), as is collagen 2a1 in the cartilage primordium (Fig. 6A, j). In murine limb bud development (18.5 dpc; hind limb) expression of *Brachyury* is evident in distinct chondrogenic cells of the forming metatarsal bones (Fig. 6B, a-c). In contrast, collagen 1a1 is expressed in the outer periosteal layer (Fig. 6B, d - f) and collagen 2a1 expression is enhanced in differentiating chondrocytes (Fig. 6B, g - i). Interestingly, like in intervertebral disc formation, the expression of *Brachyury* is only evident in chondrocyte-like cells that do not express Col 2a1 indicating that Brachyury expression is upregulated in chondrogenic cells before or after collagen 2 expression.